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Influence of charge differences in the C-terminal part of nisin on antimicrobial activity and signaling capacity

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Three mutants of the lantibiotic nisin Z, in which the Val32 residue was replaced by a Glu, Lys or Trp residue, were produced and characterized for the purpose of establishing the role of charge differences in the C-terminal part of nisin on antimicrobial activity and signaling properties. ^{1}H-NMR analyses showed that all three mutants harbor an unmodified serine residue at position 33, instead of the usual dehydroalanine. Apparently, the nature of the residue preceding the serine to be dehydrated, strongly affects the efficiency of modification. Cleavage of [Glu32,Ser33]nisin Z by endoproteinase Glu-C yielded [Glu32]nisin Z(1–32)-peptide, which has a net charge difference of -2 relative to wild-type nisin Z. The activity of [Lys32,Ser33]nisin Z against *Micrococcus flavus* was similar to that of wild-type nisin, while [Trp32,Ser33]nisin Z, [Glu32,Ser33]nisin Z and [Glu32]nisin Z(1–32)-peptide exhibited 3–5-fold reduced activity, indicating that negative charges in the C-terminal part of nisin Z are detrimental for activity. All variants showed significant loss of activity against *Streptococcus thermophilus*. The potency of the nisin variants to act as signaling molecules for auto-induction of biosynthesis was significantly reduced. To obtain mutant production, extracellular addition of (mutant) nisin Z to the lactococcal expression strains was essential.

Keywords: nisin; protein engineering; charged residue; antimicrobial activity; autoregulation.

The antimicrobial peptide nisin, which is produced by several strains of *Lactococcus lactis*, inhibits growth of a broad range of Gram-positive bacteria and, therefore, is applied as a natural preservative in various food products [1]. Nisin belongs to a group of small antimicrobial peptides, known as lantibiotics, containing dehydrated Ser and Thr residues, named dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, and intramolecular thioether ring structures called (β-methyl)-lanthionines [2, 3]. Two naturally occurring variants of nisin, named nisin A [4, 5] and nisin Z [6, 7] have been found in lactococcal strains. They differ in a single amino acid residue at position 27, this being a histidine in nisin A and an asparagine in nisin Z. The antimicrobial properties of these species are almost identical [7].

The chromosomally located nisin structural gene is followed by 10 downstream genes (*nisBTCIPRKFEG*), which are involved in nisin biosynthesis or immunity [8–14]. The maturation pathway involves post-translational modification (presumably by NisB and NisC), translocation (NisT) [8, 9] and extracellular removal of the leader peptide (NisP) [10, 11]. The proteins NisI, F, E and G are assumed to be involved in self-protection of the producer cell against the activity of nisin [12, 13]. It has been shown that nisin acts as an extracellular signal for the two-component regulatory system, consisting of histidine protein kinase NisK and the corresponding response regulator NisR. Via this signal transduction pathway, transcription of nisin genes is activated [14]. How the biosynthesis of nisin mutants will be affected by the altered signaling capacities of these mutants has not yet been studied.

The antimicrobial activity of nisin is thought to result from the formation of pores in the cytoplasmic membrane of target bacteria, through which cellular constituents are released [15–19]. Model membrane studies showed that nisin has a high anionic phospholipid specificity and it has been suggested that positively charged amino acids of nisin electrostatically interact with the target membrane [15–19]. Several modified nisin species with altered antimicrobial activities have been described [20–22]. The activity of nisin was mainly affected by modifications in the first three rings of nisin, indicating the importance of the nature of the N-terminal part. The chemically modified nisin A variant nisin A(1–32)-peptide amide, in which the two C-terminal residues are absent, was shown to exhibit similar activity to wild-type nisin [23]. In this truncated nisin, however, no net charge difference is brought about since the C-terminus is modified to an amide. To obtain more insight into the contribution of C-terminal residues for antimicrobial properties, three nisin mutants were constructed in which Val32 was modified. We introduced a positively charged Lys, a negatively charged Glu, which is rare in lantibiotics, and a Trp, which exhibits a high affinity for membranes. An additional variant, [Glu32]nisin Z(1–32)-peptide, was obtained by cleavage of [Glu32,Ser33]-nisin Z with endoproteinase Glu-C, thereby introducing an extra net negative charge in the C-terminal part of nisin. Nisin variants were purified and their structural and functional properties were

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Abbreviations. MIC, minimal inhibitory concentration; Dha, dehydroalanine; Dhb, dehydrobutyrine; RP, reverse phase.

studied. The results demonstrate that all mutants contain an unmodified Ser33 and that they display moderate or reduced antimicrobial activities, depending on the target organism. It is also shown that Val32 substitutions significantly influence the signaling potency of nisin, which has important implications for the production method of these nisin species.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and growth conditions. *Escherichia coli* MC1061 [24] was used as a recipient strain in cloning experiments [25]. To produce nisin variants, *Lactococcus lactis* strain NZ9800 [9] was used, which is a transconjugant of the plasmid-free strain MG1614 harboring a single copy of the nisin-sucrose transposon Tn*5276* [26], with a 4-bp deletion in the gene *nisA*, making it unable to produce nisin A [9]. The expression plasmid pNZ9013 [20], which was constructed by cloning a 280-bp *Pst*I–*Hin*dIII fragment carrying the wild-type *nisZ* gene under control of the *lac* promoter into the vector pNZ123, was used for site-directed mutagenesis. The *nisZ* gene was obtained from *L. lactis* NIZO 22186 [6]. Mutated *nisZ*-containing DNA fragments were cloned in expression plasmid pNZ9019, which carries a mutant *nisZ* gene with an additional internal *Pst*I restriction site [20]. The resulting plasmids, named pNZV32E, pNZV32K and pNZV32W, encoding [Glu32,Ser33]-nisin Z, [Lys32,Ser33]nisin Z and [Trp32,Ser33]nisin Z, respectively, were used to transform *E. coli* and *L. lactis* strains. *E. coli* strains were grown with aeration in 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, pH 7.2 (TY) broth at 37°C. *L. lactis* strains were grown without aeration at 30°C in M17 broth (Difco) supplemented with 0.5% (mass/vol.) sucrose. Chloramphenicol (10 μg/ml) was added to the media when appropriate. For isolation of nisin species, *L. lactis* strains were grown without aeration at 30°C in 3% sucrose, 1% peptone (Difco), 1% yeast extract (Difco), 0.2% NaCl, 0.002% $MgSO_4$, and 1% KH_2PO_4, pH 6.8 (SPYS medium). During 10-liter fermentation, the pH was maintained at 6.1 by pH-stat control using 10 M NaOH.

Micrococcus flavus DSM 1790 [20] was grown in MF broth (0.1% sucrose, 1% pepton, 0.3% meat extract, 0.2% NaCl, 0.15% yeast extract, pH 7.0), *Streptococcus thermophilus* Rs [20] in TGV broth (1% trypton, 0.3% meat extract, 0.5% yeast extract, 0.23% filtrated tomato juice, 2% glucose and 0.2% K_2HPO_4, pH 6.9).

Site-directed mutagenesis, DNA manipulation and DNA sequence analysis. Site-directed mutagenesis of the nisin Z-encoding gene was performed by two successive PCR reactions as described before [27]. Sequences of primers used for PCR are as follows: (1) 5′-GATTAAATTCTGCAGTTTGT-TAG-3′ (*Pst*I); (2) 5′-CCCTAAAAAGCTTATAAAAATAGG-3′ (*Hin*dIII); (3) 5′-GTAATTGTAGTATTCACGAAAGCAAA-TAACCAAATC-3′ [Glu32]nisin Z; (4) 5′-GTAATTGTAGTA-TTCACAAGAGCAAATAACCAAATC-3′ [Lys32]nisin Z; (5) 5′-GTAATTGTAGTATTCACTGGAGCAAATAACCAAATC-3′ [Trp32]nisin Z.

Sites of mutation are underlined and restriction enzymes used in cloning experiments are in parentheses. Plasmid isolation from *E. coli* and transformation of *E. coli* strains were carried out by established procedures [25]. To confirm the desired mutations and the integrity of the *nisZ* gene, mutant genes were sequenced by the dideoxy chain-termination method [28]. Plasmids carrying the proper mutations were introduced into *L. lactis* NZ9800 by electroporation [29]. The integrity of the constructed plasmids was confirmed by digestion with the restriction enzymes indicated. Restriction enzymes and other DNA-modifying enzymes were purchased from Life Technologies, Inc. or U.S. Biochemical Corp. and used as recommended by the manufacturers.

Purification and characterization of nisin mutants. For purification of mutant nisin peptides, expression strains were grown to early stationary phase in SPYS medium. In order to enhance the production level, 0.05 μg/ml wild-type nisin A was added to the growing culture to ensure that transcription of the nisin biosynthetic genes and immunity genes was fully activated [14]. Nisin species were purified by hydrophobic-interaction chromatography using Fractogel TSK butyl 650-M (Tosohaas), followed by preparative reverse-phase (RP)-HPLC as described previously [20]. The mutant species were eluted by a linear gradient of 27–31% 90% aqueous acetonitril, 0.07% trifluoroacetic acid (buffer B) in 10% aqueous acetonitril, 0.1% trifluoroacetic acid (buffer A), using a flow rate of 8.8 ml/min. Elution was monitored by determining the $A_{220\,nm}$. Absolute amounts of nisin were estimated by determining the specific absorbance at 220 nm as described before [20]. Mutant species were checked for purity by analytical RP-HPLC using a 4.5 mm×250 mm widepore RP column (Biorad) and a gradient of 25%–28% buffer B in buffer A. [Ser33]Nisin Z was isolated as a minor component from 10 liter culture supernatant of a nisin Z-producing strain and it was purified to homogeneity as described above. To obtain the nisin variant [Glu32]nisin Z(1–32)-peptide, 1 mg purified [Glu32,Ser33]nisin Z was proteolytically cleaved by 20 μg endoproteinase Glu-C (ICN), which is known to cleave behind Glu and Asp residues, in 0.05 M sodium phosphate, pH 6.6, at 30°C for 3 h. Purification of the cleavage product was performed by preparative RP-HPLC as described above.

1-dimensional and 2-dimensional ^{1}H-NMR analyses of mutant nisin species. Structural analyses of the nisin peptides were performed by ^{1}H-NMR. NMR spectra were taken on a Bruker AM400 spectrometer operating at 400.13 MHz for protons, interfaced to an Aspect 3000 computer. The samples were dissolved in 90% H_2O/10% D_2O (9 mg/ml) and the pH adjusted to 3.5 (pH meter reading). All experiments were performed at 25°C. Spectra were referenced to external 3-(trimethylsilyl)-2,2,3,3-tetradeutero-propionic acid (BDH). The solvent resonance was suppressed by saturation during the relaxation delays. TOCSY spectra were taken using a MLEV-17 mixing sequence with a duration of 60–80 ms. For the NOESY spectra, a mixing time of 400 ms was used. In the NOESY experiments, the solvent resonance was also irradiated during the mixing period. TOCSY spectra were used for the identification of typical amino acid patterns and NOESY spectra were used for the sequential assignment essentially as described elsewhere [23, 30].

Determination of antimicrobial activities. Antimicrobial activities of mutant nisin Z species were determined by a plate diffusion assay using *M. flavus* DSM 1790 as indicator organism. Minimal inhibitory concentration (MIC) values were determined against the indicator strains *M. flavus* DSM 1790 and *S. thermophilus* Rs by use of previously described procedures [20]. The MIC assays were performed in triplicate.

Determination of signaling capacities. For quantitative determination of induction capacities of mutant peptides, *L. lactis* NZ3900 was used, which contains a single copy of the *nisRK* genes on the chromosome [31], harboring plasmid pNZ8008, which contains a nisin promoter fragment fused to the promoterless reporter gene *gusA* of *E. coli* [14]. *L. lactis* NZ3900 was cultered in M17 broth, supplemented with 0.5% (mass/vol.) glucose at 30°C. After induction of the cells with a range of concentrations of wild-type or mutant nisin, β-glucuronidase activities were measured as described [14]. The signaling capacities of mutants were determined by comparing the dose-response lines of wild-type nisin Z and the mutant species.

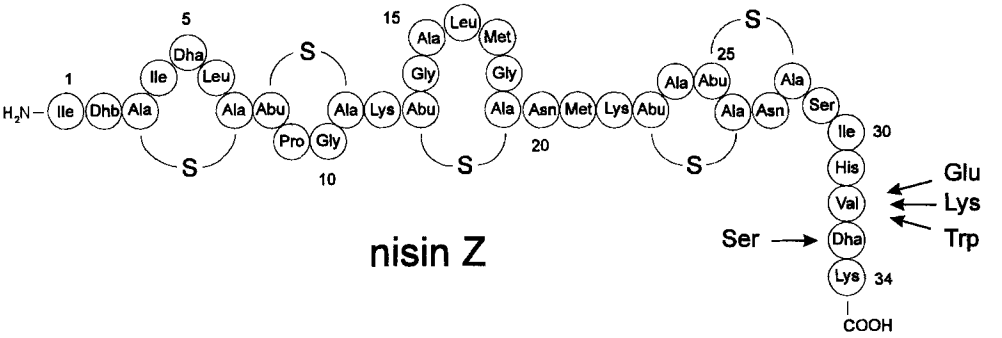

Fig. 1. Primary structure of nisin Z. The modifications, which are described in this paper are indicated by arrows. Ser indicates a lack of modification at residue 33 in the mutants [Glu32,Ser33]nisin Z, [Lys32,Ser33]nisin Z and [Trp32,Ser33]nisin Z. Dha, dehydroalanine; Dhb, dehydrobutyrine.

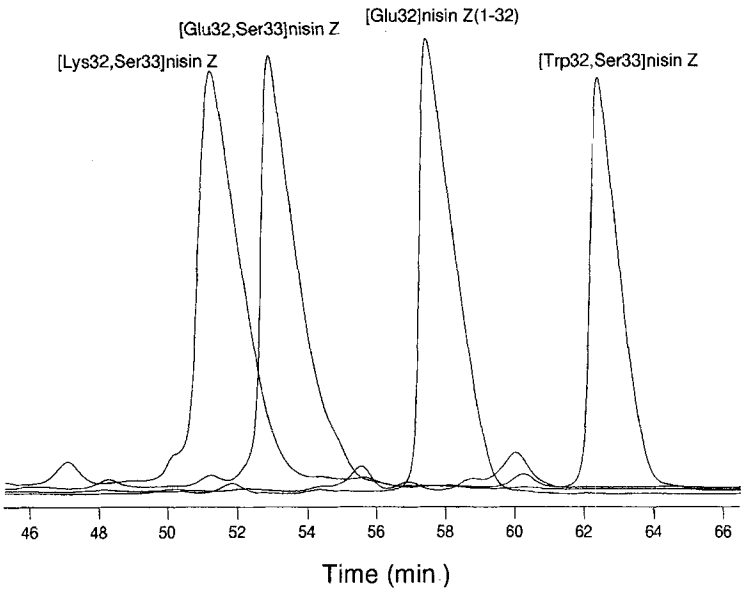

Fig. 2. Analytical RP-HPLC analyses of purified [Glu32,Ser33]nisin Z, [Lys32,Ser33]nisin Z, [Trp32,Ser33]nisin Z and [Glu32]nisin Z(1–32)-peptide.

RESULTS

Production of mutant nisin Z species. Three mutant nisin genes were constructed encoding the negatively charged Glu, the positively charged Lys or the aromatic Trp at position 32 (Fig. 1). Purification of the mutant nisin species yielded 35 mg [Glu32,Ser33]nisin Z, 15 mg [Lys32,Ser33]nisin Z and 5 mg [Trp32,Ser33]nisin Z from 10 litres fermentation broth, which is low in comparison with wild-type-nisin-producing strains, which yield 100 mg on average.

Since we aimed at studying the effects of charged residues in the C-terminus of nisin, purified [Glu32,Ser33]nisin Z was treated with endoproteinase Glu-C and the cleavage product [Glu32]nisin Z(1−32)-peptide was also purified. Due to the presence of a negatively charged Glu32 and the removal of the positively charged Lys34, the difference in net charge of this variant in comparison with wild-type nisin is −2.

Purified nisin species were examined by analytical RP-HPLC (Fig. 2). Wild-type nisin Z is not indicated in Fig. 2, since the retention times of [Glu32]nisin Z(1−32)-peptide and wild-type nisin Z were found to be almost equal. As expected, the substitution of Val32 for the hydrophobic Trp resulted in a longer retention time. The presence of the hydrophilic Glu or Lys in nisin Z resulted in a shorter retention time.

Structural characterization of mutant nisin Z species. The purified peptides were subjected to ^{1}H-NMR spectroscopy to verify the presence of the Glu, Lys or Trp residues at position 32, and to see whether there were any other structural differences relative to wild-type nisin (Fig. 3). The absence of the vinyl resonances of dehydroalanine (dha) residue 33 (5.7 ppm) in the spectra of the mutants indicates that formation of Dha33 did not occur. The ^{1}H-NMR spectrum of [Trp32,Ser33]nisin Z shows an additional indole NH resonance of Trp at 10.09 ppm and resonances from the aromatic ring protons of Trp at 7.1 and 7.6 ppm.

The structures of [Glu32,Ser33]nisin Z, [Glu32]nisin Z(1−32)-peptide and [Trp32,Ser33]nisin Z were further examined by two-dimensional NMR techniques. For this purpose TOCSY and NOESY spectra of the mutants were recorded and the spectra were assigned as described for wild-type nisin (Table 1) [23, 30]. TOCSY spectra were used for spin-system identification; sequential assignment and structural characterization of the lanthionine ring structures was achieved with the corresponding NOESY spectra. Comparison of the assignment of the modified peptides with that of wild-type nisin Z [20] showed that the differences of chemical shifts were small (< 0.05 ppm), with the exception of residues located near the substituted residue 32. A

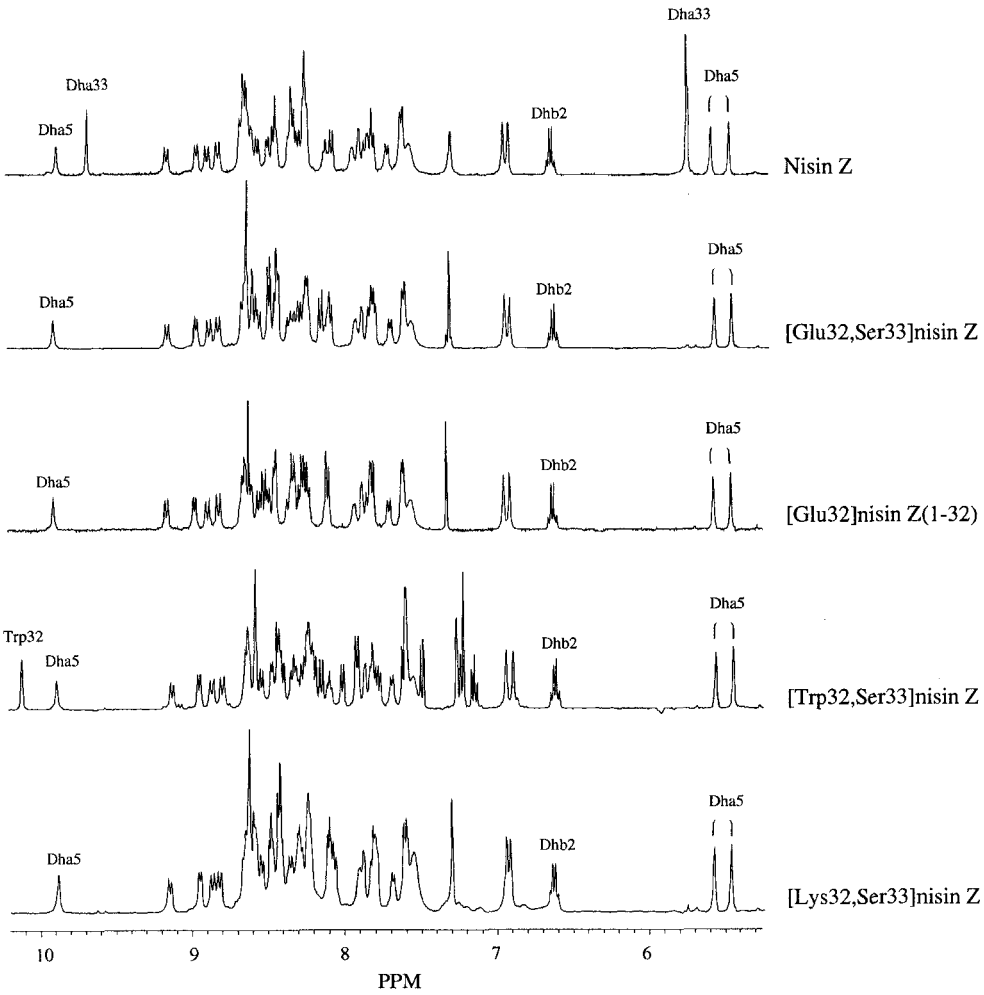

Fig. 3. ^{1}H-NMR spectra of wild-type nisin Z, [Lys32,Ser33]nisin Z, [Glu32,Ser33]nisin Z, [Glu32]nisin Z(1–32)-peptide and [Trp32,Ser33]-nisin Z. Proton resonances of relevant residues are indicated.

Table 1. Proton resonance assignments of [Glu32,Ser33]nisin Z, [Glu32]nisin Z(1–32)-peptide and [Trp32,Ser33]nisin Z. Eperimental conditions: 10% D_2O, 90% H_2O, pH 3.5, 25 °C. Proton resonances differing more than 0.05 ppm from those of wild-type nisin Z are indicated. For assignment of the ^{1}H-NMR spectrum of wild-type nisin Z, see [20].

Residue	Chemical shift			
	$N^{\alpha}H$	$C^{\alpha}H$	$C^{\beta}H$	other
[Glu32,Ser33]nisin Z				
Glu32	8.43	4.49	1.99, 2.14	$C^{\gamma}H_2$ 2.46
Ser33	8.49	4.49	3.90	
Lys34	8.15	4.28	1.82, 1.87	$C^{\gamma}H_2$ 1.43 $C^{\delta}H_2$ 1.77 $C^{\epsilon}H_2$ 3.02 $N^{\zeta}H_3^+$ 7.56
[Glu32]nisin Z(1−32)-peptide				
His31	8.51	4.79	3.25	C^2H 8.62 C^5H 7.32
Glu32	8.32	4.30	1.96, 2.16	$C^{\gamma}H_2$ 2.44
[Trp32,Ser33]nisin Z				
Ile30	8.01	4.07	1.73	$C^{\gamma}H_2$ 1.07, 1.30 $C^{\gamma'}H_3$ 0.81 $C^{\epsilon}H_3$ 0.65
His31	8.43	4.83	3.10, 3.22	C^2H 8.58 C^5H 7.22
Trp32	8.20	4.71	3.24, 3.32	$C^{\beta}H_2$, $C^{\gamma}H_2$, C^2H 7.26, C^4H 7.62, C^6H 7.22, C^7H 7.50, NH 10.09, C^5H 7.1
Ser33	8.15	4.39	3.77	
Lys34	7.92	4.14	1.71, 1.82	$C^{\gamma}H_2$ 1.39 $C^{\delta}H_2$ 1.71 $C^{\epsilon}H_2$ 3.01 $N^{\zeta}H_3^+$ 7.56

correct formation of thioether bridges and unsaturated amino acids, except for the formation of Dha33, was established in all variants. In the spectra of [Glu32,Ser33]nisin Z and [Trp32, Ser33]nisin Z proton resonances of Glu and Trp residues, respectively, could be identified. Also resonances of Ser33 were assigned. The replacement of Val32 by a Glu residue resulted in small differences of the chemical shifts of Lys34, whereas substitution of Val32 for a Trp affected the chemical shifts of

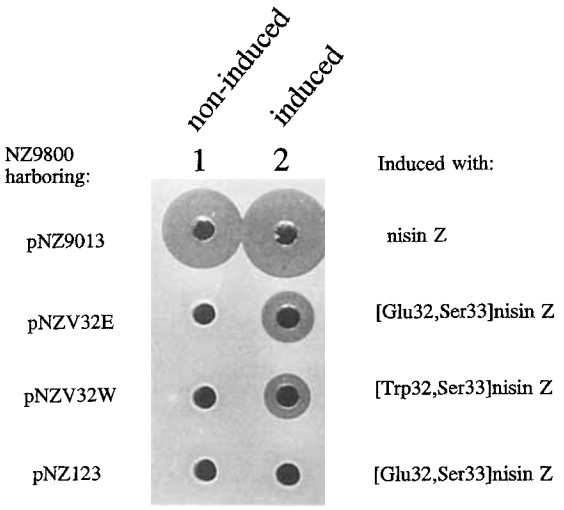

Fig. 4. Agar diffusion assay using *M. flavus* as indicator organism. Inhibition zones were obtained by spotting supernatant of NZ9800 strains harboring the different vectors which are indicated on the left. Column 1, cultures were grown without addition of inducing peptides. Column 2, to induce nisin production, 0.03 μg/ml peptide indicated on the right was added to the culture. For the [Lys32,Ser33]nisin Z producing strain, similar results were observed.

Table 2. Antimicrobial activities of nisin Z variants. The net charge difference of the mutants relative to nisin Z is indicated. Standard errors were less than 20% for each given value.

Nisin variant	Charge	Minimal inhibitory concentration	
		M. flavus	*S. thermophilus*
		μg/l	
Nisin Z	–	11	6
[Ser33]Nisin Z	0	11	6
[Trp32,Ser33]Nisin Z	0	30	20
[Lys32,Ser33]Nisin Z	+1	11	50
[Glu32,Ser33]Nisin Z	−1	45	100
[Glu32]Nisin Z(1−32)	−2	55	150

the residues 30−34, most likely due to the ring current shift (Table 1). In the TOCSY spectrum of [Glu32]nisin Z(1−32)-peptide the patterns of Ser33 and Lys34 were absent and residues 31 and 32 showed differences in chemical shift as compared to wild-type nisin Z.

Since the variations of the chemical shifts of the nisin mutants, relative to those of nisin Z are rather small, the NMR data do not provide indications for major structural changes of the nisin mutants in aqueous environment due to the Val32 substitutions.

Antimicrobial activities of the nisin mutants. The growth-inhibiting activities of the modified nisins towards *M. flavus* and *S. thermophilus* were determined (Table 2). Replacement of Val32 by a Lys did not reduce the activity against *M. flavus*, while a Glu at this position led to fourfold reduction. Removal of residues 33 and 34 of the latter mutant further decreased activity, indicating that negative charges in the C-terminus interfere with nisin antimicrobial activity. The presence of a Glu as well as a Lys lowered the activity against *S. thermophilus*. Introduction of a Trp on position 32, which causes no charge differences, resulted in 2−3-fold lower antimicrobial activity against *M. flavus* and *S. thermophilus*, respectively.

Table 3. Signaling capacities of nisin Z variants. Values were determined as described under Materials and Methods. The signaling capacity of wild-type nisin Z was taken as 100% value. Standard errors were less than 20% for each given value.

Nisin variant	Signaling capacity
	%
Nisin Z	100
[Ser33]Nisin Z	100
[Trp32,Ser33]Nisin Z	9
[Lys32,Ser33]Nisin Z	28
[Glu32,Ser33]Nisin Z	27
[Glu32]Nisin Z(1−32)	8

Signaling capacities of mutant nisin species. To obtain information about the potency of the nisin mutants to autoregulate (mutant) nisin production, supernatant of the lactococcal expression strain NZ9800, harboring different pNZ-derived vectors was used in an agar diffusion assay with *M. flavus* as indicator organism. When the NZ9800 strains were grown without addition of extracellular inducing peptide, only production of wild-type nisin Z by the strain harboring pNZ9013, could be seen. Mutant production by the cells harboring the plasmids pNZV32E, pNZV32W or pNZV32K was not observed (Fig. 4; column 1).

For production of nisin Z mutants, the expression strain NZ9800 can in some cases be grown in the presence of a low amount (0.03 μg/ml) of wild-type nisin A, to help to induce the expression of the essential biosynthesis and immunity genes. We find that for production of [Glu32,Ser33], [Lys32,Ser33] and [Trp32,Ser33]nisin Z, addition of nisin to the medium was essential. To see whether this was caused by the incapacity of the nisin variants to activate NisK-dependent signal transduction, for instance due to a disturbed interaction with the sensor protein NisK, 0.03 μg/ml purified [Glu32,Ser33]nisin Z or [Trp32, Ser33]nisin Z was added to the culture medium and supernatant was tested for activity. Addition of the purified mutants could activate mutant production, demonstrating that the mutants are able to act as signaling peptides (Fig. 4; column 2). The induced NZ9800 cultures, producing wild-type or mutant nisin, were subsequently diluted 1:5 in fresh medium, to determine whether the cells were able to continue nisin production when the inducing peptide was diluted. The production level of wild-type nisin Z remained high, demonstrating the efficient autoregulatory mechanism of the wild-type-producing strain. In contrast, the production of [Glu32,Ser33]nisin Z and [Trp32, Ser33]nisin Z decreased rapidly and after subculturing several times the cultures became non-producing again (not shown).

We further investigated whether uninduced NZ9800 cells, harboring pNZV32E or pNZV32W, started to produce mutant peptide after several generation times, reasoning that accumulation of mutant peptide in the supernatant might eventually lead to efficient induction of mutant nisin biosynthesis. However, after repeatingly subculturing the strains, the activity in the supernatant remained negligible (not shown). These results indicate that the cells cannot produce sufficient mutant peptide in the extracellular medium that is required for triggering mutant production, which might be due to a lower biosynthesis rate, involving modification, translocation and maturation of the mutant peptides. Furthermore, the potency of the mutants to act as inducing factor might also be of importance, since a reduction of

the induction capacities means that a relatively high concentration of mutant peptide is required in the medium for triggering mutant production. Therefore, the potency of the mutant nisin species to activate transcription by acting as an extracellular signal for the histidine protein kinase NisK was quantitatively determined, using β-glucuronidase reporter assays. Substitution of Val32 by a Glu, Lys or Trp resulted in significant reduction of the signaling capacity of nisin (Table 3). Since all mutants contain an unmodified Ser33, we also examined the induction capacity of [Ser33]nisin Z, which was isolated from a wild-type nisin Z-producing culture. This peptide displayed induction of the *gusA* gene at a level similar to wild-type nisin Z, indicating that the presence of an unmodified Ser33 does not affect the induction capacity. After proteolytic cleavage of [Glu32,Ser33]-nisin Z with endoproteinase Glu-C only 8% of the signaling capacity of nisin Z was left.

DISCUSSION

Nisin Z variants, harboring a positively charged Lys, a negatively charged Glu or an aromatic Trp residue at position 32, were purified and the effects of these C-terminal alterations on structure, antimicrobial activities and signaling properties of nisin were studied.

^{1}H-NMR analyses of the purified mutants showed that they all contain an unmodified Ser33. It has been shown before that, during biosynthesis of nisin, low amounts of [Ser33]nisin are produced [32], indicating that some nisin molecules escape modification on position 33. However, in the mutants containing a Glu, Lys or Trp residue on position 32, dehydration of Ser33 was completely disturbed. Probably, the residues preceding Ser33 cause electrostatic or steric hindrance of dehydrating enzymes, putatively NisB. Remarkably, residues Lys12 and Lys22 in nisin do not disturb dehydration of the following Thr residues. Also, the structurally related lantibiotic subtilin contains a Glu at position 4, directly followed by Dha5 [33]. Introduction of a Trp at position 1 in nisin Z, resulted in partial dehydration of the following Thr2, yielding both [Trp1,Dhb2]nisin Z and [Trp1, Thr2]nisin Z [21]. These data indicate that the position of the residue to be dehydrated is of importance for modification, and that the C-terminal Ser33 is a relatively poor substrate for dehydrating enzymes.

So far, modifications in the first three ring structures of nisin were found to have the largest effects on activity [20, 21]. Furthermore, it was found that N-terminal nisin A fragments, such as nisin A(1−21) and nisin A(1−19), obtained by chemical synthesis [34] or nisin A(1−20), obtained by prolonged exposure of wild-type nisin A to chymotrypsin [35], exhibited decreased antimicrobial activity. The C-terminal modifications that were studied here, also resulted in significant reduction of the activity against *M. flavus* and *S. thermophilus*. Since the absence of Dha33 does not impair nisin activity, loss of activity of the mutants must have been caused by the valine substitutions. The mutant [Trp32,Ser33]nisin Z displayed threefold reduced activity against *M. flavus* and *S. thermophilus*. By introducing a negative charge on position 32, the activity against both organisms was strongly reduced. Removal of Ser33 and Lys34, thereby increasing the net negative charge, led to further loss of activity. Since it was shown that the activity of nisin A(1−32)-peptide amide is very similar to that of nisin A itself [23], the loss of activity of [Glu32]nisin Z(1−32)-peptide was probably due to charge differences and not to the absence of the last two residues. Since lantibiotics often posses a C-terminal Lys or an amide, resulting in a net charge of zero at the C-terminal amino acid residue, it was postulated before that missing negative charges might be important for activity [36]. However, increase of positive charges in the C-terminal part of nisin, as in [Lys32, Ser33]nisin Z, did not improve the activity. Although there was no reduction of the activity against *M. flavus*, we observed eightfold reduced activity against *S. thermophilus*. It has been reported before that the activity of nisin was not enhanced after replacement of the residues Asn27 and His31 by Lys [22]. Obviously, the effects of the modifications on activity are highly dependend on the target organism. The *in vivo* activity of nisin is proposed to require several steps, including diffusion through the cell wall, interaction with the cytoplasmic membrane, penetration of the lipid bilayer and formation of pores. Modifications of nisin might influence one or more of these processes, resulting in overall changes of activity and/or specificity of mutants. As reported before, nisin has a high affinity for anionic lipids of Gram-positive bacteria [15−19]. Reasonably, the reduced activity of [Glu32,Ser33]nisin Z and [Glu32]nisin Z(1−32)-peptide might be explained by repulsion of negatively charged components present in the cell wall or the cytoplasmic membrane of bacteria. Investigations on interactions of Glu or Lys containing nisin mutants with model membranes are in progress. The results indicate that, compared to wild-type nisin Z, the capacity of [Glu32,Ser33]nisin Z to bind to lipid vesicles is significantly lower using vesicles with a high content of anionic lipids, which suggests that the Glu residue decreases membrane affinity by ionic repulsion (data not shown).

The autoregulatory system of nisin production is known to be very efficient, requiring only few nisin peptides for triggering nisin production [14]. Production of the mutants [Glu32,Ser33]-nisin Z, [Lys32,Ser33]nisin Z and [Trp32,Ser33]nisin Z by the strain NZ9800, however, could only be activated by addition of inducing peptides. This can be explained by a combined effect of a less efficient biosynthesis and reduced signaling capacities of the nisin variants. We assume that the concentration of signaling peptides in the medium must exceed a threshold level to trigger nisin production and that the production level of these mutant species was too low for induction, resulting in a non-producing strain. In addition, a relatively high extracellular concentration of the mutants is required for efficient induction, since it was demonstrated that the potency of the mutants to act as signaling peptides was significantly reduced. The regulatory system of the NZ9800 strains producing [Glu32,Ser33]nisin Z, [Lys32,Ser33]nisin Z or [Trp32,Ser33]nisin Z is reminiscent of inducing systems of *Lactobacillus plantarum* [37], *Lactobacillus sake* [38], or *Carnobacterium piscicola* [39] producing the bacteriocins plantaricin, sakacin, and carnobacteriocin, respectively. In Bac$^-$ cultures of these strains, bacteriocin production can only be activated by addition of sufficient amounts of inducing peptide to the culture. A producing strain can subsequently be turned into a non-producing one by diluting the culture, thereby decreasing the concentration of inducing peptide [37−39].

The results discussed above show that the C-terminal part of nisin contributes significantly to antimicrobial activity and signaling potency of nisin. In addition, the modified peptides constructed in this study provide a valuable tool for mechanistic studies using model membrane systems. Further characterization of these C-terminal nisin variants will therefore be focussed on membrane interactions.

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REFERENCES

1. Delves-Broughton, J., Blackburn, P., Evans, R. E. & Hugenholtz, J. (1996) Applications of the bacteriocin nisin, *Anthonie van Leeuwenhoek 69*, 193–202.
2. Gross, E. & Morell, J. L. (1971) The structure of nisin, *J. Am. Chem. Soc. 93*, 4634–4635.
3. Jung, G. (1991) Lantibiotics: a survey, in *Nisin and novel lantibiotics* (Jung, G. & Sahl, H. G., eds) pp. 1–31, ESCOM Science Publishers, Leiden.
4. Buchmann, G. W., Banerjee, S. & Hansen, J. N. (1988) Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic, *J. Biol. Chem. 263*, 16260–16266.
5. Kaletta, C. & Entian, K.-D. (1989) Nisin, a peptide antibiotic: cloning and sequencing of the *nisA* gene and posttranslational processing of its peptide product, *J. Bacteriol. 171*, 1597–1601.
6. Mulders, J. W. M., Boerrigter, I. J., Rollema, H. S., Siezen, R. J. & de Vos, W. M. (1991) Identification and characterization of the lantibiotic nisin Z, a natural nisin variant, *Eur. J. Biochem. 201*, 581–584.
7. De Vos, W. M., Mulders, J. W. M., Siezen, R. J., Hugenholtz, J. & Kuipers, O. P. (1993) Properties of nisin Z and distribution of its gene, *nisZ*, in *Lactococcus lactis*, *Appl. Environ. Microbiol. 59*, 213–218.
8. Engelke, G., Gutowski-Eckel, Z., Hammelman, M. & Entian, K.-D. (1992) Biosynthesis of the lantibiotic nisin: genome organization and membrane localization of the NisB protein, *Appl. Environ. Microbiol. 58*, 3730–3743.
9. Kuipers, O. P., Beerthuyzen, M. M., Siezen, R. J. & de Vos, W. M. (1993) Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*; Requirement of expression of the *nisA* and *nisI* genes for development of immunity, *Eur. J. Biochem. 216*, 281–291.
10. Van der Meer, J. R., Polman, J., Beerthuyzen, M. M., Siezen, R. J., Kuipers, O. P. & de Vos, W. M. (1993) Characterization of the *Lactococcus lactis* nisin A operon genes *nisP* encoding a subtilisin-like serine protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis, *J. Bacteriol. 175*, 2578–2588.
11. Siezen, R. J., Rollema, H. S., Kuipers, O. P. & de Vos, W. M. (1995) Homology modeling of the *Lactococcus lactis* leader peptidase NisP and its interaction with the precursor of the lantibiotic nisin, *Protein Eng. 8*, 117–125.
12. Engelke, G., Gutowski-Eckel, Z., Kiesau, P., Siegers, K., Hammelmann, M. & Entian, K.-D. (1994) Regulation of nisin biosynthesis and immunity in *Lactococcus lactis* 6F3, *Appl. Environ. Microbiol. 60*, 814–825.
13. Siegers, K. & Entian, K.-D. (1995) Genes involved in immunity to the lantibiotic nisin produced by *Lactococcus lactis*, *Appl. Environ. Microbiol. 60*, 814–825.
14. Kuipers, O. P., Beerthuyzen, M. M., De Ruyter, P. G. G. A., Luesink, E. J. & de Vos, W. M. (1995) Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction, *J. Biol. Chem. 270*, 27299–27304
15. Sahl, H.-G. (1991) Pore formation in bacterial membranes by cationic lantibiotics, in *Nisin and novel lantibiotics* (Jung, G. & Sahl, H.-G., eds) pp. 347–358, ESCOM Science Publishers, Leiden.
16. Abee, T., Gao, F. H. & Konings, W. N. (1991) The mechanism of action of lantibiotic nisin in artificial membranes, in *Nisin and novel lantibiotics* (Jung, G. & Sahl, H.-G., eds) pp. 373–385, ESCOM Science Publishers, Leiden.
17. Driessen, A. J. M., van den Hooven, H. W., Kuiper, W., van den Kamp, M., Sahl, H.-G., Konings, R. N. H. & Konings, W. N. (1995) Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles, *Biochemistry 34*, 1606–1614.
18. Gao, F. H., Abee, T. & Konings, W. N. (1991) Mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome *c* oxidase-containing proteoliposomes, *Appl. Environ. Microbiol. 57*, 2164–2170.
19. Demel, R. A., Peelen, T., Siezen, R. J., de Kruijff, B. & Kuipers, O. P. (1996) Nisin Z, mutant nisin Z and lacticin 481 interactions with anionic lipids correlate with antimicrobial activity – A monolayer study, *Eur. J. Biochem. 235*, 267–274.
20. Kuipers, O. P., Rollema, H. S., Yap, W. M. G. J., Boot, H. J., Siezen, R. J. & de Vos, W. M. (1992) Engineering dehydrated amino acid residues in the antimicrobial peptide nisin, *J. Biol. Chem. 267*, 24340–24346.
21. Kuipers, O. P., Bierbaum, G., Ottenwälder, G., Dodd, H. M., Horn, N., Metzger, J., Kupke, T., Gnau, V., Bongers, R., van den Boogaard, P., Kosters, H., Rollema, H. S., de Vos, W. M., Siezen, R. J., Jung, G., Götz, F., Sahl, H.-G. & Gasson, M. J. (1996) Protein engineering of lantibiotics, *Anthonie van Leeuwenhoek 69*, 161–169.
22. Rollema, H. S., Kuipers, O. P., Both, P., de Vos, W. M. & Siezen, R. J. (1995) Improvement of solubility and stability of the antimicrobial peptide nisin by protein engineering, *App. Environ. Microbiol. 61*, 2873–2878.
23. Chan, W. C., Bycroft, B. W., Lian, L.-Y. & Roberts, G. C. K. (1989) Isolation and characterization of two degradation products derived from the peptide antibiotic nisin, *FEBS Lett. 252*, 29–36.
24. Casadaban, M. J. & Cohen, S. N. (1980) Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*, *J. Mol. Biol. 143*, 179–207.
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor NY.
26. Rauch, P. J. G. & de Vos, W. M. (1992) Characterization of the novel nisin-sucrose conjugative transposon Tn*5276* and its insertion in *Lactococcus lactis*, *J. Bacteriol. 174*, 1280–1287.
27. Kuipers, O. P., Boot, H. J. & de Vos, W. M. (1991) Improved site-directed mutagenesis method using PCR, *Nucleic Acids Res. 19*, 4558.
28. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors, *Proc. Natl Acad. Sci. USA 74*, 5463–5467.
29. Wells, J. M., Wilson, P. W. & Le Page, R. W. F. (1993) Improved cloning vectors and transformation procedure for *Lactococcus lactis*, *J. Appl. Bacteriol. 74*, 629–636.
30. Slijper, M., Hilbers, C. W., Konings, R. N. H. & van de Ven, F. J. M. (1989) NMR studies of lantibiotics. Assignment of the ^{1}H-NMR spectrum of nisin and identification of interresidual contacts, *FEBS Lett. 252*, 22–28.
31. De Ruyter, P. G. G. A., Kuipers, O. P., Beerthuyzen, M. M., van Alen-Boerrigter, I. & de Vos, W. M. (1996) Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*, *J. Bacteriol. 178*, 3434–3439.
32. Rollema, H. S., Both, P. & Siezen, R. J. (1991) NMR and activity studies of nisin degradation products, in *Nisin and novel lantibiotics* (Jung, G. & Sahl, H.-G., eds) pp. 123–130, ESCOM Science Publishers, Leiden.
33. Liu, W. & Hansen, J. N. (1992) Enhancement of the chemical and antimicrobial properties of subtilin by site-directed mutagenesis, *J. Biol. Chem. 267*, 25078–25085.
34. Wakamiya, T., Fukase, K., Kitazawa, M., Fujita, H., Kubo, A., Maeshiro, Y. & Shiba, T. (1990) Lanthionine peptide nisin: Study of structure-activity relationship, in *Peptides* (Rivier, J. & Marshall, G., eds) pp. 60–64, ESCOM Science Publishers, Leiden, The Netherlands.
35. Chan, W. C., Leyland, M., Clark, J., Dodd, H. M., Lian, L.-Y., Gasson, M. J., Bycroft, B. W. & Roberts, G. C. K. (1996) Structure-activity relationships in the peptide antibiotic nisin: antibacterial activity of fragments of nisin, *FEBS Lett. 390*, 129–132.
36. Kupke, T. & Götz, F. (1996) Post-translational modifications of lantibiotics, *Anthonie van Leeuwenhoek 69*, 139–150.
37. Diep, D. B., Havarstein, L. S. & Nes, I. F. (1995) A bacteriocin-like peptide induces bacteriocin synthesis in *Lactobacillus plantarum*, *Mol. Microbiol. 18*, 631–639.
38. Eijsink, V. G. H., Brurberg, M. B., Middelhoven, P. H. & Nes, I. F. (1996) Induction of bacteriocin production in *Lactobacillus sake*, *J. Bacteriol. 178*, 2232–2237.
39. Saucier, L., Poon, A. & Stiles, M. E. (1995) Induction of bacteriocin in *Carnobacterium piscicola* LV17, *J. Appl. Bacteriol. 78*, 684–690.